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THE APPLICATION OF POLYACRYLAMIDE GEL ELECTROPHORESIS TO THE CH--ETC(U)
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EDGEWOOD ARSENAL, ABERDEEN PROVING GROUND, MD.

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THE APPLICATION OF POLYACRYLAMIDE GEL ELECTROPHORESIS TO THE CHARACTERIZATION OF CHOLINESTERASE ENZYMES USED IN DETECTOR KITS

by

Alan Goodman
Lawrence M. McCormack
Hermann Martens

Directorate of Development and Engineering

January 1977

DEPARTMENT OF THE ARMY
Headquarters, Edgewood Arsenal
Aberdeen Proving Ground, Maryland 21010

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Vertical disc gel electrophoresis on polyacrylamide gel tubes has been applied to the separation and identification of cholinesterases from commercially available sources. Cholinesterase from horse serum, bovine erythrocyte, and electric eel was fractionated using the Davis method for human serum proteins. The fractions were visualized with a protein stain and two cholinesterase substrates, indoxyl acetate and indoxyl butyrate. Each enzyme had a distinctive pattern of protein

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20. ABSTRACT (Continued)

bands. The eel and bovine enzymes, which have high specific activities, showed fewer bands than did the horse enzyme which has lower specific activity (lower purity). All three enzymes hydrolyzed indoxyl acetate giving colored bands. Only the horse enzyme, however, gave colored bands with indoxyl butyrate, which is in agreement with the known substrate specificities of cholinesterases. The small sample size required, the wide variations possible in gel pore size, and buffer system composition permit optimization of sensitivity and resolution for the particular enzyme being investigated and make the method useful for analytical quality control, preparative scale separations, and characterization of small amounts of enzyme from intelligence sources.

PREFACE

The work described in this report was authorized under Task 1W762710AD2902, Detection and Identification Applications. This work was started in March 1973 and completed in June 1974

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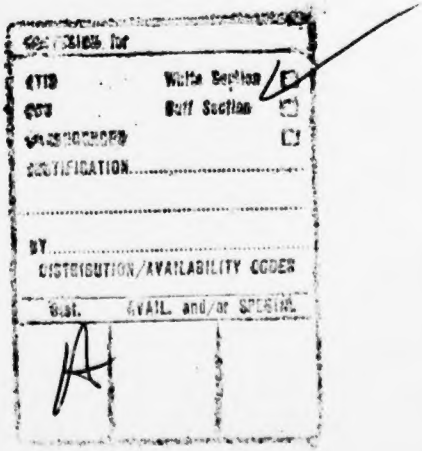

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THE APPLICATION OF POLYACRYLAMIDE GEL ELECTROPHORESIS TO THE CHARACTERIZATION OF CHOLINESTERASE ENZYMES USED IN DETECTOR KITS

I. INTRODUCTION.

Field detection of anticholinesterase agents is based on the agents' ability to prevent enzymatic hydrolysis of a chromogenic substrate by a cholinesterase-impregnated paper disc. The appearance of the blue chromophore is an indication that nerve agents are not present, while no color change indicates that an agent is present. The M15- and M18-type kits use horse serum cholinesterase.[1] Similar Canadian devices use bovine erythrocyte cholinesterase.[2] Cholinesterase from other sources is also being considered for detection applications. These detector tickets are prepared by impregnating the paper discs with a known volume of cholinesterase solution and drying under vacuum. Their usefulness is then dependent on the cholinesterase activity remaining on the impregnated ticket. This activity is determined, for the US tickets, by a titrametric assay at constant pH using acetylcholine as the substrate.[3] It has been found, however, that some tickets which assay within specification do not give a good chromogenic substrate response. Some further characterization of the enzyme is indicated. The commercially available cholinesterases. horse serum, bovine erythrocyte, and electric eel are not high purity materials. They are known to contain other esterases as well as nonenzyme protein. The effect of the other materials upon substrate response, inhibitor action, and ticket wettability and stability is not known and cannot always be measured by titrametric enzyme assay. There also exists a need for identification and characterization of activity, substrate specificity, and inhibitor sensitivity for enzymes obtained from foreign detectors. In these cases, there is frequently an insufficient quantity of enzyme to perform the usual kinetic assays of activity.[3]

A rapid, efficient technique for high resolution separation of proteins from complex mixtures has recently been developed. This technique, called polyacrylamide gel electrophoresis (PAGE), works on the principle that proteins of different charge and size migrate through a controlled pore polymer gel at different rates when a voltage is applied across the gel. It was developed by Ornstein[4] and Davis[5] for clinical screening of human serum proteins but has found wide applications in enzyme and protein chemistry. In this method the separating or running gels, consisting of acrylamide monomer, a crosslinking agent, and a catalyst are prepared by polymerization in glass tubes, 4 mm ID. A spacer or stacking gel is then polymerized on top of each running gel; then another gel containing the protein sample is polymerized over the stacking gels. A voltage is then applied across the gel tube, and each protein migrates through the gel at a rate determined by its own mobility which is, in turn, determined by its size and charge. After electrophoresis, the gels are removed from the glass tubes and stained with a blue-black dye which has a strong affinity for protein but none for the gel matrix material. The excess dye is then washed away leaving the stained protein bands clearly visible in the transparent gel. This method, or a modification of it, has been used by several workers for purification and characterization of cholinesterases[6-8] and as an assay of homogeneity of cholinesterases purified by other methods.[9-11] This report describes the use of PAGE techniques to characterize commercial cholinesterase preparations and identify "fingerprint" patterns of the different enzymes which would be of use in identification of enzymes from foreign items.

II. METHODS AND MATERIALS.

A. Materials.

1. Chemicals.

Acrylamide, N,N - methylenebisacrylamide (BIS), N,N,N′,N′-tetramethyethylenediamine (TEMED), and ammonium persulfate were purchased from Bio-Rad Laboratories, Richmond, CA. Tris (hydroxymethyl) aminomethane (TRIS), glycine, riboflavin, sucrose, indoxyl, acetate, and Amido Black 10B (also called naphthol blue black) were purchased from Sigma Chemical Co., St. Louis, MO. Indoxyl butyrate was purchased from Koch Light, Colinbrook, England. Other chemicals were reagent grade. Water used was double distilled.

2. Enzymes.

Horse serum cholinesterase and bovine erythrocyte acetylcholinesterase were purchased from Nutritional Biochemical Co., Cleveland, Ohio, and electric eel acetylcholinesterase was purchased from Sigma Chemical Co., St. Louis, MO. The horse and eel enzymes were lyophilized salt-free powder while bovine erythrocyte enzyme contained salts, buffers, and a protective colloid, gelatin, in the commercial preparation.

3. Equipment.

Electrophoresis was performed using Model 150 gel electrophoresis cell purchased from Bio-Rad Laboratories, Richmond, CA (pictured in figure 1). The gels were prepared in a Bio-Rad Model 700 gel preparation apparatus shown in figure 2. A Bio-Rad Model 170 diffuser destainer was used to wash the excess dye from the stained gels (figure 3).

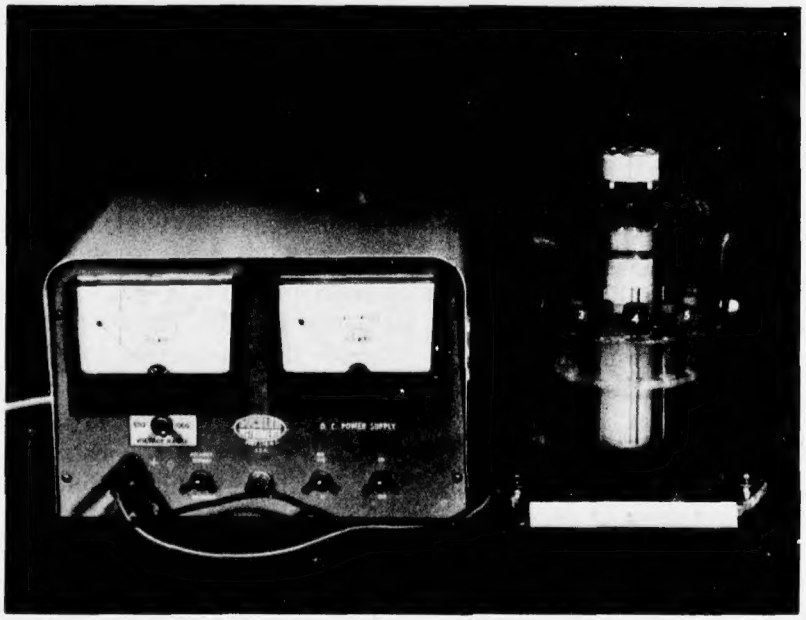


Figure 1. Gel Electrophoresis Cell with Power Supply

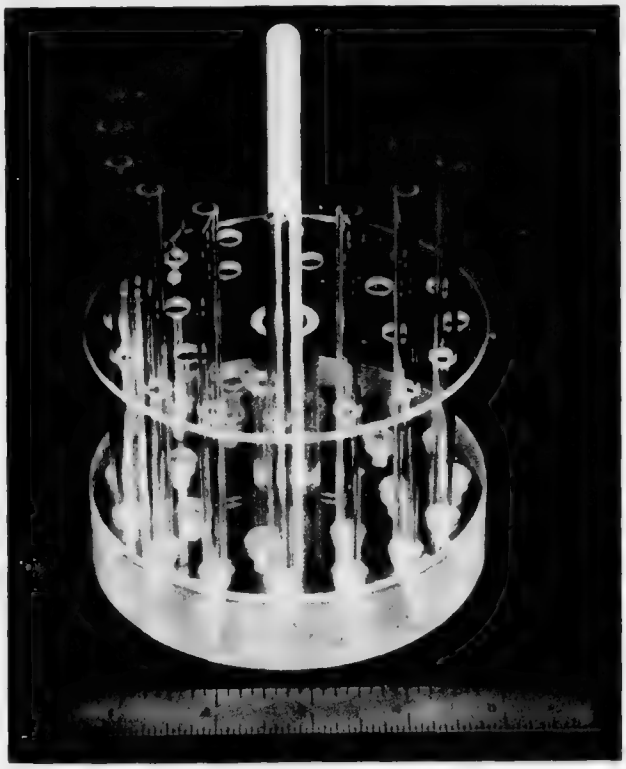


Figure 2. Gel Preparation Apparatus

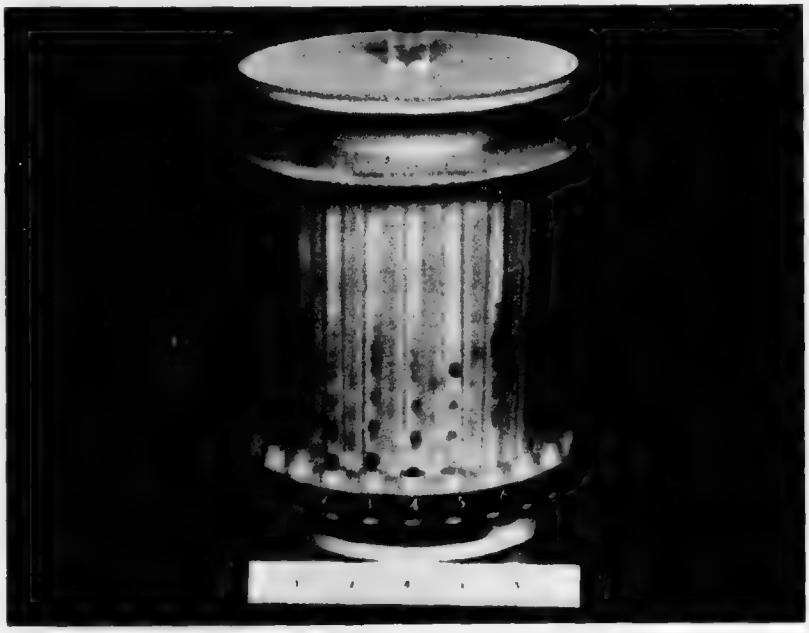


Figure 3. Diffusion Destainer for Acrylamide Gels

B. Experimental Procedures.

The experimental techniques are described by Smith[12] and are based on the method of Davis[5]

1. Stock Solutions.

The following solutions were prepared.

a. Small Pore Gel Monomer.

Acrylamide	–	28.0 gm
BIS	–	0.775 gm
Distilled water	–	to make 100 ml

b. Large Pore Gel Monomer.

Acrylamide	–	10.0 gm
BIS	–	2.5 gm
Distilled water	–	to make 100 ml

c. Small Pore Buffer pH 8.9.

lN-hydrochloric acid	–	48 ml
Tris buffer	–	36.6 gm
TEMED	–	0.23 ml
Distilled water	–	to make 100 ml

The exact amount of hydrochloric acid added is that required to bring the pH to 8.9.

d. Large Pore Buffer pH 6.7.

lN-hydrochloric acid	–	48 ml
Tris buffer	–	5.98 gm
TEMED	–	0.46 ml
Distilled water	–	to make 100 ml

Again the exact amount of hydrochloric is determined by the pH requirement.

e. Reservoir Buffer pH 8.3.

Glycine	–	28.8 gm
Tris	–	6.0 gm
Distilled water	–	to make 1000 ml

f. Catalyst.

Ammonium persulfate	–	0.14 gm
Distilled water	–	to make 100 ml

g. Catalyst.

Riboflavin	–	0.004 gm
Distilled water	–	to make 100 ml

h. Marker Dye.

Bromophenol blue	–	0.001 gm
Distilled water	–	to make 100 ml

i. Protein Stain.

Amido black 10B	–	1 gm
7% Acetic acid	–	to make 100 ml

j. Substrate.

Indoxyl acetate	–	0.035 gm
Acetone	–	2.5 ml
Ligorine (bp 100°-115°C)	–	47.5 ml

This solution is prepared by dissolving the substrate in the acetone which is then added slowly to the ligorine with stirring.

k. Substrate.

Indoxyl butyrate	–	0.035 gm
Acetone	–	2.5 ml
Ligorine (bp 100°-115°C)	–	47.0 ml

This solution is prepared in a similar manner to indoxyl acetate solution.

l. Sucrose Solution.

Sucrose – 40 gm

Distilled water – to make 100 ml

Solutions a and b are kept refrigerated and are considered good for 2 months. Solutions f and g are stored refrigerated, protected from light, and prepared fresh weekly. Solutions j and k are prepared fresh immediately before use. All other solutions are stored at room temperature and are considered to be stable for 6 months.

2. Preparation of Gels.

The gel tubes are covered at one end with paraffin film and placed, covered end down, in the gel preparation unit (figure 2). The following gel solution is then prepared and 1.8 ml is added with a syringe into each gel tube keeping the syringe needle below the liquid surface to minimize air bubble formation.

a. Running (Separating) Gel Solution.

Component	Amount used for preparing 10 gel tubes ml
Small-pore buffer solution	2.5
Small-pore monomer solution	5.0
Ammonium persulfate solution	10.0
Distilled water	2.5

Immediately after filling the tubes, each gel is overlaid with 30 µl of water from a syringe, with the needle touching the inner wall of the tube just above the gel line. The tubes are allowed to stand for 15 minutes. The gel solution begins to polymerize almost immediately after being mixed. It is therefore important to apply the water overlay quickly to prevent a curved meniscus at the surface of the polymerized gel. A curved meniscus would cause broadening and gross distortion of the protein bands as they migrate into the running gel. After completion of polymerization, the water overlay is drained off, and the running gel surface is washed once with spacer gel/sample gel solution and is drained again. Then the spacer gel is prepared by adding 0.150 ml of this spacer gel/sample gel solution and is overlayed with water, as above. The composition of this gel is as follows:

b. Spacer Gel/Sample Gel.

Component	Amount used for preparing 10 gel tubes ml
Large-pore buffer solution	0.5
Large-pore monomer solution	1.0
Riboflavin solution	0.5
Sucrose solution	2.0

The rack containing the gels is then placed directly in front of a fluorescent light (desk lamp) until polymerization is complete (normally 30 to 60 minutes). Complete polymerization is indicated by turbidity in the gel. Again, the water is drained and the tube is rinsed with gel solution. The sample gel which consists of the gel solution above to which is added a volume of enzyme solution containing 0.9 to 1.5 units* of enzyme is then added. The total volume of sample gel is 1.0 to 1.2 ml. In a typical run, two to four tubes are prepared for each enzyme being assayed. The sample gels are difficult to polymerize and require longer times than do the spacer gels. When polymerization is complete, the paraffin films are removed and the gel tubes are assembled in the electrophoresis cell (figure 1). The reservoir buffer is prepared by diluting the stock solution (solution 5) by 1:10. The lower reservoir is filled with 1100 ml of this working solution. The upper reservoir contains 400 ml of this buffer along with 2 drops of bromophenol blue dye solution. This dye migrates faster than any of the protein components and serves as a "front" marker. The instrument is then connected to the power supply, upper reservoir to the cathode and lower reservoir to the anode. The jacket around the lower buffer contains water circulating from a constant temperature bath at 2° to 4°C. When the lower reservoir buffer reaches this temperature, the run is started.

3. Electrophoresis.

The initial voltage is set to maintain a current of 1 ma per gel tube and is kept at this value until the bromophenol blue band enters the running gel. The voltage is then increased to maintain a current of 5 ma per tube. Operations at currents higher than 5 ma per tube can cause thermal stress distortions and shearing in the gel. This current is maintained until the bromophenol blue discs have migrated to about 1 cm from the lower end of the tubes. The tubes are then removed from the apparatus and the gels are removed from the tubes. To aid in removing the gels from the tubes, a syringe with a long thin (22 gauge) needle is used. The syringe is filled with water and the needle is carefully inserted between the gel and inner tube wall. While slowly injecting water, the tube is turned keeping the needle against the inner tube wall rimming the gel. This must be done slowly and carefully to avoid scarring or breaking the gel.

When most of the gel has been lubricated this way, it will readily slide out of the tube. It is then immediately placed in a test tube for staining.

4. Staining and Destaining.

The gels are stained for protein with Amido Black 10B and for cholinesterase activity with indoxyl acetate and indoxyl butyrate. The Amido Black solution is prepared by filtering the 1% stock solution, described above, then diluting 1:10 with 7% acetic acid. This solution is then added to the test tubes containing the gels and is allowed to stand for 30 minutes. The gels are removed and washed with 7% acetic acid and then placed in the diffusion destainer filled with 7% acetic acid which is stirred with a magnetic stirrer overnight.

The indoxyl acetate and indoxyl butyrate staining is done by adding the stock solution, described above, into the test tubes containing the gels and allowing to incubate in a water bath at 37°C until reaction is visually evident (usually 30 to 60 minutes). The gels are then washed and placed in the destainer along with the Amido Black stained gels.

* One unit of cholinesterase is defined as that amount required to hydrolyze 1 μmole of acetycholine per minute at 25°C, pH 8.

III. RESULTS.

The Amido Black stained gels show that each enzyme has a definite, distinctive pattern of protein bands, as illustrated in figure 4. Repeated runs of the same enzymes give identical results. The higher purity of the eel enzyme is indicated by its small protein bands as compared to the horse and bovine enzymes. The indoxyl acetate stained gels (figure 5) all show cholinesterase activity. They all show the same narrow band 13 to 14 mm from the top (cathodic) end of the gel while the bovine enzyme shows an additional diffuse band at 19 to 22 mm from the cathodic end. Of the indoxyl butyrate stained gels (figure 6), only the horse serum enzyme gel shows any cholinesterase activity. This is expected since all of the enzymes are known to hydrolyze indoxyl acetate while only the horse enzyme hydrolyzes the butyrate. The bovine and eel enzymes, the so-called acetyl or "true" cholinesterases, have very low activity to butyrate esters while the horse serum enzyme, a nonspecific cholinesterase, has enough activity toward both esters to show strong staining. Another conformation of the higher purity of eel enzyme as compared to horse is that the eel enzyme shows one band with both protein stains and substrate stain while the horse, which has multiple bands (8 to 10) with protein stain, has only one cholinesterase band indicated by substrate stains.

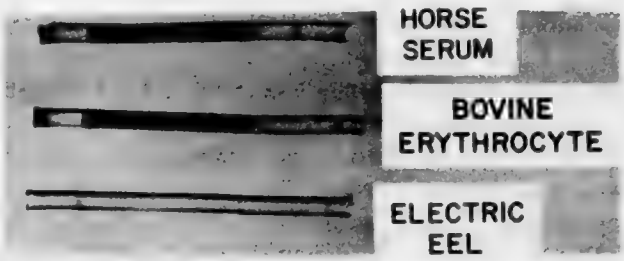


Figure 4. Electrophoresis of Cholinesterase Enzymes Stained with Amido Black (Protein Stain)

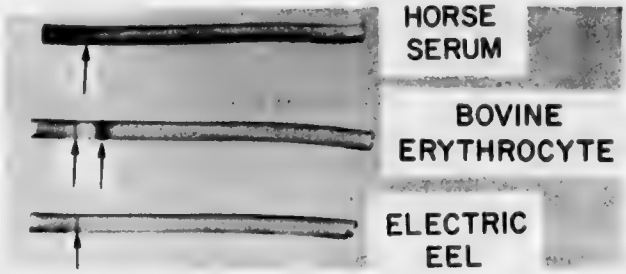


Figure 5. Electrophoresis of Cholinesterase Enzymes Stained with Indoxyl Acetate Substrate

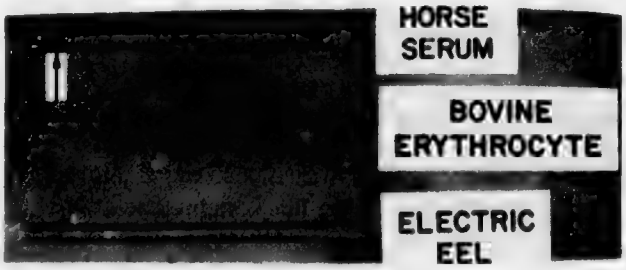
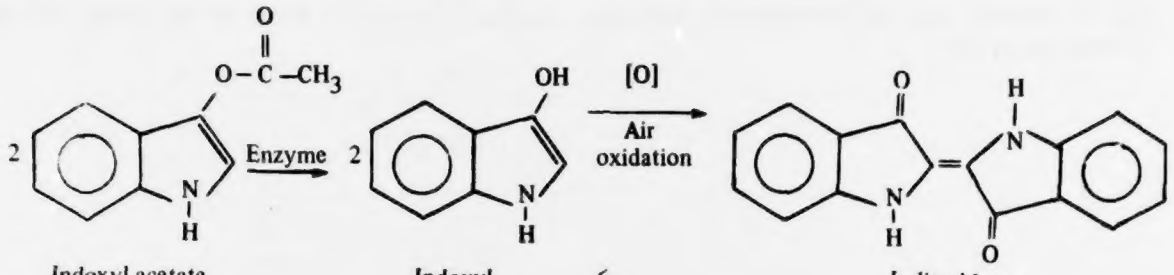


Figure 6. Electrophoresis of Cholinesterase Enzymes Stained with Indoxyl Butyrate Substrate

The faint narrow band near the anodic end (at the right on figure 4) on the Amido Black stained gels is bromophenol blue tracker dye and should not be mistaken for a protein band.

IV. DISCUSSION.

Indoxyl esters have been used for many years to visualize cholinesterases.[13] The esters are hydrolyzed by cholinesterase to free indoxyl which is colorless but is quickly air-oxidized to indigo blue as shown by the following reactions:



Indoxyl acetate Indoxyl 6 Indigo blue

The substance responsible for the blue color is the same regardless of which ester, acetate, or butyrate is used.

PAGE has been developed, within recent years, into a powerful analytical tool in enzyme and protein chemistry. It is a rapid, a sensitive, and an inexpensive method for separating and identifying the various components and fractions of enzyme preparations and is well suited for analysis and quality control of sensitive, specific detection reagents employing enzymes. Operating conditions of the gel system can easily be varied to suit the particular enzyme. The ionic mobility of a given protein species is determined by the pore diameter in the gel which is controlled by the concentrations of monomer (acrylamide) and cross-linking agent (BIS). Optimum separation for a given protein mixture can be achieved and, in fact, the relationship between ionic mobilities and pore size has been quantitated and molecular weight determinations have been done, using PAGE techniques.[14] The choice of pH and buffer components is also important since the sensitivity and resolving power of PAGE is due, in part, to a concentration phenomenon in which the individual protein components are concentrated or "stacked" between moving boundaries of discontinuous buffer phases in the gel.[14] This effect allows the use of very small amounts of proteins and makes the protein concentration in the gel bands independent of initial protein concentration.

The buffer system used in these experiments was designed for optimum separation of human serum proteins fractions[5] but has found wide application. It is not necessarily the optimum one for cholinesterase purifications or characterization though. A treatment of multiphase buffer systems which permits design of an optimum system for any given separation on an analytical or preparative scale has recently been presented.[15-17] Given the desired pH and temperature, one can devise a buffer system to separate the desired protein mixture knowing the pK's and ionic mobilities of the proteins and buffers.

V. CONCLUSIONS.

1. Polyacrylamide Gel Electrophoresis is a useful analytical tool for separation and characterization of the components of commercially available cholinesterase preparations. It is an

attractive method for production and quality control of enzyme preparation for use in future detector kit applications and could be used as an analytical method when preparing a specification for purchasing enzymes for kit manufacture.

2. The small samples of enzyme required make this technique useful for characterization of enzyme or protein obtained in the field or from intelligence sources. Many compounds, which react with enzymes or proteins to give colored products, can be used as stains to distinguish one species from another.

3. The wide range of gel parameters, pH value, and buffer systems available makes this a versatile and an inexpensive technique applicable to many areas in the detection and identification field.

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